Regulated Interaction of Protein Kinase Cδ with the Heterogeneous Nuclear Ribonucleoprotein K Protein*

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The heterogeneous nuclear ribonucleoprotein (hnRNP) K protein recruits a diversity of molecular partners that are involved in signal transduction, transcription, RNA processing, and translation. K protein is phosphorylated in vivo and in vitro by inducible kinase(s) and contains several potential sites for protein kinase C (PKC) phosphorylation. In this study we show that K protein is phosphorylated in vitro by PKCδ and by other PKCs. Deletion analysis and site-directed mutagenesis revealed that Ser302 is a major K protein site phosphorylated by PKCδ in vitro. This residue is located in the middle of a short amino acid fragment that divides the two clusters of SH3-binding domains. Mutation of Ser302 decreased the level of phosphorylation of exogenously expressed K protein in phorbol 12-myristate 13-acetate-treated COS cells, suggesting that Ser302 is also a phosphorylation site for PKCδ in vivo. In vitro, PKCδ binds K protein via the highly interactive KI domain, an interaction that is blocked by poly(C) RNA. Mutation of Ser302 did not alter the K protein-PKCδ interaction in vitro, suggesting that phosphorylation of this residue alone is not sufficient to alter this interaction. Instead, binding of PKCδ to K protein in vitro and in vivo was greatly increased by K protein phosphorylation on tyrosine residues. The ability of PKCδ to bind and phosphorylate K protein may serve not only to alter the activity of K protein itself, but K protein may also bridge PKCδ to other K protein molecular partners and thus facilitate molecular cross-talk. The regulated nature of the PKCδ-K protein interaction may serve to meet cellular needs at sites of active transcription, RNA processing and translation in response to changing extracellular environment.

The hnRNP1 K protein has a diverse repertoire of molecular partners that are involved in signal transduction and gene expression. K protein binds with RNA, single-stranded, and double-stranded DNA, and it associates with a number of transcriptional activators and repressors, including TATA-binding protein (1). K protein also interacts with tyrosine (2) and serine/threonine kinases (3–5) as well as the proto-oncoprotein Vav (3, 6, 7). The diverse molecular interaction of K protein may account for the observations that K protein can both increase (8, 9) and decrease (10–12) gene transcription. For example, on one hand K protein synergies with TATA-binding protein to increase transcription from the c-myc promoter CT element (8), whereas on the other it represses C/EBPβ-mediated transcription of the agp gene (11) and inhibits Sp-1-mediated activation of the neuronal nicotinic acetylcholine receptor promoter (12). As one of the constituents of the hnRNP particle, K protein may be involved in the processing of pre-mRNA (13, 14). K protein shuttles between the nucleus and cytoplasm and therefore could serve as a vehicle that is involved in RNA transport (15). K protein-mediated silencing of 15-lipoxygenase mRNA (16) represents an example of K protein involvement in the regulation of transcription. Involvement of K protein in translational processes is further supported by its association with the elongation factor 1α (1). The association of K protein with tyrosine kinases (2, 3) and with Vav (6, 7) may reflect involvement of K protein in signal transduction. Alternatively, the Vav- and/or tyrosine kinases-K protein interaction may regulate K protein transcriptional and/or translational activity. Considering the diversity of K protein molecular interactions, it is not surprising that new reports are emerging implicating K protein involvement in viral processes. For example, K protein has been shown to functionally interact with hepatitis C virus core protein (17) and to regulate translation of the human papillomavirus type 16 L2 mRNA (18).

K protein is made up of modular domains that bind different molecular partners. For example, the three KI domains are thought to mediate nucleic acid binding (19, 20), whereas the two clusters of SH3-binding sites recruit the proto-oncoprotein Vav (3) and the Src-class of tyrosine kinases (2). The latter sites are contained within the same region that binds several transcriptional repressors, such as Zik1 (21), as well as the global regulator of anterior-posterior patterning, Eed (22). This module is adjacent to a nuclear shuttling domain, KNS (15), that may mediate direct coupling of K protein to a bona fide nuclear/cytoplasmic shuttling transporter(s). Finally, a domain near the C terminus recruits an interleukin-1-responsive kinase that phosphorylates K protein in a nucleic acid-dependent fashion (3, 4). Although K protein is already known to contain a number of binding domains, it seems that there are potentially others that remain to be identified. The abundant and ubiquitous expression of K protein, its multimodular structure, its potential to oligomerize via its two different dimerization do-
mains, and its apparent involvement in a wide range of processes responsible for transcription, translation, and signal transduction suggest that K protein may act as a scaffold or docking platform. Alternatively, K protein may be a multifunctional factor that is involved in processes that are not directly related. In either scenario the function of K protein is likely to be regulated by post-translational modification and by cognate nucleic acid motifs.

K protein is phosphorylated in vivo and in vitro on serine and threonine residues (5, 23). At least in part, this phosphorylation is mediated by an associated kinase(s) that can respond to treatment of cells with interleukin-1 and other agents (4, 5). Thus, K protein phosphorylation is likely to play a key role in the regulation of its activity. This postulate is supported by the observation that the binding of K protein to poly(C) in vitro is diminished by phosphorylation (23). Moreover, in hepatocytes following systemic administration of lipopolysaccharide into rats, there was a complete dissociation of the transcriptional factor C/EBPβ from K protein (11), a process that may reflect phosphorylation of K protein. Analysis of the K protein amino acid sequence reveals a number of potential phosphorylation sites by casein kinase II, protein kinase C (PKC), and tyrosine kinases. Indeed, in vitro, K protein is an excellent substrate for casein kinase II (24). However, casein kinase II is typically a constitutively active enzyme (25), making its role in the inducible phosphorylation of K protein in vivo less likely than the role of inducible serine/threonine kinases such as the PKC family of enzymes.

The PKC family of enzymes transduce intracellular signals that regulate many different intracellular processes (26). This heterogenous family of enzymes is divided into three classes based on their Ca2+ and lipid requirements (27). The conventional PKCs (α, β, and γ) require phosphorylserine, diacylglycerol, or phorbol 12-myristate 13-acetate (PMA), and Ca2+; the novel subgroup of PKCs (δ, ε, η, μ, and θ) are Ca2+-independent but require the other two co-factors; and finally the least understood subgroup of PKCs (z and λ) are both Ca2+- and diacylglycerol-independent. Among the PKC isoenzymes, PKCδ has unique properties that suggest a functional connection to K protein. First, PKCδ phosphorylates elongation factor 1α (28), a factor that binds K protein (29). Second, K protein interacts with Src-tyrosine kinases, a class of enzymes that phosphorylate PKCδ (30). Third, K protein contains several potential PKC phosphorylation sites. Because K protein contains sites for PKC phosphorylation and because PKCδ and K protein share molecular partners, in this study we explored the possibility that K protein is a PKCδ substrate.

MATERIALS AND METHODS

Cell Line—The murine thymoma EL-4 6.1 C10 (31) and human thymoma Jurkat cell line was grown in suspension. Cells were grown at 37 °C in complete RPMI 1640 medium supplemented with 5% fetal calf serum or Fetal Clone (Hyclone), 2 mM glutamine, 50 μM β-mercaptoethanol, 100 units/ml penicillin, 0.01% streptomycin, and humidified with 7/93% CO2/air gas mixture.

Protein A-Sepharose was obtained from Amersham Pharmacia Biotech. Polyclonal anti-K protein antibody 54 was made in rabbits as described previously (4). Monoclonal antiphosphotyrosine and polyclonal anti-PKCδ antibody was purchased from Santa Cruz and anti-PKCδ monoclonal antibody was purchased from Transduction Laboratories. Bacteriophages expressed and spleen PKCδ were produced as described previously (33, 34).

List of Buffers—The following buffers were used: PKC phosphorylation buffer, 50 mM Tris, pH 7.5, 10 mM β-mercaptoethanol, 1 mM PMA, 10 mg phosphatidylserine, 18.75 mM ATP, and 3.7 mM cAMP of γ32P[ATP; PKC binding buffer, 50 mM Tris, pH 7.5, 10 mM β-mercaptoethanol, and 1 mM PMA; Lck phosphorylation buffer, 20 mM Tris-Cl, pH 7.5, 10 mM MnCl2, 100 μM ATP, 1 mM diithiothreitol, and 0.1% Triton X-100; Escherichia coli extraction buffer, 50 mM Tris-Cl, pH 8.0, 2 mM EDTA, 0.5 mM diithiothreitol, 10 mg/ml lysozyme, 0.01% Triton X-100, and 0.5 mM phenylmethylsulfonyl fluoride; standard binding buffer, 5 mM Tris, pH 7.5, 1 mM EDTA, 25 mM NaCl, and 0.05% Nonidet P-40; HKMT buffer, 10 mM Hespe, pH 7.5, 2 mM MgCl2, 0.1% Triton X-100, and 100 mM KCl; washing buffer, 5 mM Tris-Cl, pH 7.5, 175 mM NaCl, 1.0 mM EDTA, and 1.0% Nonidet P-40; SDS-loading buffer, 60 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, and 1% β-mercaptoethanol; TBE buffer, 50 mM Tris base, 5 mM EDTA, pH 7.5, 150 mM NaCl, and 0.05% Tween 20; and IP buffer, 150 mM NaCl, 5 mM Tris-Cl, pH 7.5, 5 mM EDTA, 0.5% Nonidet P-40, and 1.0% Triton X-100.

Extraction of Cytoplasmic and Nuclear Proteins—Nuclear and cytoplasmic extracts were prepared by a modified version of the method of Dignam et al. (35) as described previously (5). In addition to 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, lysis, extraction, and dilution buffers contained the following phosphatase inhibitors (all from Sigma): 30 mM p-nitrophenyl phosphate, 10 mM NaF, 0.1 mM NaVO4, 0.1 mM Na3MoO4, and 10 mM β-glycerophosphate. Protein content was measured using the DC method (Pierce).

K Protein Plasmids Constructs—GST-K, GST-K3, GST-K, GST-K10, GST-K12, GST-K13, and GST-K14 were constructed as described (3). For the GST-K13 deletion mutant, polymerase chain reaction was used to introduce a BanHI site immediately upstream of Met96 and to introduce a stop codon followed by an EcoRI site downstream of Val337. GST-K31 was created by cloning this excised fragment into pGEX-KX. For the GST-KAPB deletion mutant, pGEX-K was cut with PvuII and BglII, then filled in with Klenow fragment, and religated to create the GST-KAPB construct. For Flag-K for mammalian expression, K protein was excised from pMK1-21 with EcoRI and SalI and then ligated into p18Flag that had been cut with EcoRI and XhoI, creating the recombinant Flag-K. The point mutations of Ser302 to Ala or Glu in GST-K, GST-K31, and Flag-K were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene). All plasmids were purified by CsCl gradient before use in transient transfections, and the mutations were confirmed by automated sequencing using the DyeDeoxy Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA).

Synthesis and Purification of GST-K Constructs—GST fusion proteins were expressed in either BRL or BL21 (DE3) pLysS cells (Novagen, Madison, WI) using a modified manufacturer’s protocol. Transformed cells were grown until they reached an absorbance at 600 nm of 0.6 and then were treated with 1.0 mM isopropyl-β-D-thiogalactoside. Following freezing and thawing, pellets were resuspended in E. coli extraction buffer and sonicated. Fusion proteins were recovered in the supernatant after centrifugation at 14,000 rpm for 30 min (4 °C). GST-K and GST-K deletion mutants were then purified on a glutathione column as described previously (3).

In Vitro Transcription and Translation—In vitro transcription and translation was performed using the TNT T7 Quick Coupled Transcription/Translation System as per the manufacturer’s protocol (Promega, Madison, WI).

Binding to Beads Bearing GST-K Proteins—Binding to beads was carried out by mixing glutathione beads with the standard binding buffer containing a given protein or RNA at 4 °C. After extensive washes, proteins were eluted from the beads by boiling in 1× SDS-loading buffer (36) and were then loaded on a 10% SDS gel and autoradiographed.

Transient Transfections—COS cells were grown in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum to approximately 60–75% confluency in 100-mm diameter dishes and were transfected using SuperFect Transfection Reagent as per the manufacturer’s protocol (Qiagen Inc., Santa Clarita, CA).

RESULTS

K Protein Is Phosphorylated in Vitro by PKCδ—K protein is phosphorylated in vitro and in vivo on serine residues by a kinase(s) with which it forms a complex (5, 23). Analysis of K protein amino acid sequence reveals a number of potential sites for phosphorylation by PKC. To test whether K protein is a substrate for this class of enzymes, purified bacterially expressed full-length GST-K fusion protein was phosphorylated in solution by purified porcine spleen PKCδ in the presence of...
The reaction mixture was separated by SDS-PAGE, GST-K protein bands were cut out, and radioactivity was measured. Rates of phosphate incorporation were plotted as a function of GST-K protein concentration (A), and 1/V was plotted as a function of 1/[GST-K] in B. The y axis intercept of the double-reciprocal plot (B) is 1/V_{max} = 77 pmol min^{-1} or V_{max} = 0.013 pmol/min/45.5 ng PKÇ - 286 pmol/min/mg PKÇ. The x axis intercept of the double-reciprocal plot is 1/K_{m} = -0.53 nm^{-1} or K_{m} = 1.9 nm.

Phosphorylation of K Protein in Vivo—PKÇ-A series of GST-K deletion mutants (Fig. 2A) (3) was used to identify a domain that is phosphorylated by PKÇ. Glutathione beads bearing either full-length GST-K or one of the deletion mutants were phosphorylated by baculovirus-expressed PKÇ, and the phosphorylation reaction was carried out as in Fig. 1. Afterward, the beads were washed once with 200 ul of standard binding buffer. 32P-labeled proteins were eluted from the beads by boiling in SDS-loading buffer and then separated by SDS-PAGE. The gels were stained (Coomassie) and autoradiographed (32P)/B. Molecular mass markers are shown in kDa.

We used the above approach to determine whether K protein is a substrate for other PKC isoforms. Beads bearing different GST-K protein mutants (Fig. 3A) were phosphorylated by a partially purified mixture of PKÇa, PKÇb, and PKÇg. As with PKÇc, GST-K and GST-K31 were phosphorylated by PKÇa, PKÇb, and PKÇg, whereas GST-K7 (a.a. 318–464) was not phosphorylated at all, and the level of phosphorylation of the internal deletion mutant that lacks the proline-rich SH3-binding domains, GST-KA-PB (a.a. 288–321 deleted), was a very poor substrate for PKÇc (data not shown).

PMA-inducible Phosphorylation of K Protein in Vivo—K pro-
Phosphorylation of K Protein

**Fig. 3. In vitro phosphorylation of K protein Ser^{302} by PKCa, β, and γ.** 20 μl of glutathione beads bearing either full-length K protein or one of the K protein mutants fused to GST (A) were phosphorylated by PKCa, β, and γ purified from bovine brain (46) under conditions described for PKCδ. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography (B and C) as in Fig. 2.

protein is phosphorylated in vivo on serine residues (5). Thus, to determine whether Ser^{302} can also be phosphorylated in vivo, we compared the levels of phosphorylation of wild-type Flag-K and of point mutant Flag-K^{302A} (Ser^{302} → Ala) that were co-expressed with HA-PKCδ in COS cells. Transfected cells were metabolically labeled with [32P]orthophosphate and were then treated with or without 10^{-7} M PMA for 1 h. Following treatment, cells were harvested, and cytoplasmic and nuclear extracts were prepared as described previously (5). Equal amounts of extracts were precipitated with either pre-immune or immune anti-K protein serum (antibody 54). The immunoprecipitates were separated by SDS-PAGE and were then electrotransferred to Immobilon-P membrane (Millipore, Bedford, MA) and were analyzed by autoradiography (Fig. 4, [32P]) and by Western blotting using anti-K protein serum (Fig. 4, aK). The autoradiograph revealed that in the cytoplasm the constitutive level of phosphorylation of Flag-K was higher than that of Flag-K^{302A}. In the nuclear fraction, phosphorylation of Flag-K was PMA-inducible, whereas phosphorylation of Flag-K^{302A} was not. In both the nucleus and the cytoplasm, the level of Flag-K PMA-inducible phosphorylation was higher than the level of Flag-K^{302A} phosphorylation in PMA-treated cells. Unlike the exogenously expressed Flag-K proteins, the levels of PMA-inducible phosphorylation of endogenous K protein (Fig. 4, lower band in the autoradiograph marked K), in the Flag-K and Flag-K^{302A} transfected cells were similar. These experiments provide evidence that Ser^{302} can be phosphorylated in vitro, a reaction that may, in part, be mediated by PKCδ and/or other PKC isoenzymes.

**K Protein Binds PKCδ through Its Highly Interactive Domain**—The low K_{m}, for the phosphorylation of GST-K by PKCδ (Fig. 1) suggests that the two proteins may bind one another with a high enough affinity to form detectable complexes. To test such a possibility, beads bearing GST or GST-K fusion proteins (Fig. 5) were mixed with baculovirus expressed PKCδ, and after binding and washing, bound proteins were eluted from the beads by boiling in loading buffer. Proteins eluted from the beads were separated by SDS-PAGE and electrotransferred onto Immobilon-P membrane. Membranes were immunostained with anti-K protein monoclonal antibody 2-5 and/or other PKC isoenzyme antibodies. To ensure that beads contained similar levels of GST fusion proteins, another gel was stained with Coomassie. These results showed that beads bearing GST-K protein, but not beads bearing GST alone, pulled down PKCδ, indicating that K protein binds PKCδ in vitro. Several GST-K deletion mutants were used to map the K protein domain that binds PKCδ in vitro. GST-K13 (a.a. 1–337) and GST-K31 (a.a. 7–10) bound PKCδ as effectively as the full-length GST-K protein, whereas deletion mutants GST-K7 (a.a. 318–464), GST-K10 (a.a. 318–382), GST-K12 (a.a. 1–209), and GST-K14 (a.a. 406–464) did not bind PKCδ at all. The deletion mutant that lacks the two clusters of SH3-binding domains, GST-KΔPB (deleted a.a. 288–321 fragment), bound PKCδ weakly. These results indicate that the domain of K protein that binds
PKCδ in vitro is contained within the a.a. 240–337 region and that the proline-rich region may be important in this interaction. The a.a. 240–337 K protein domain interacts with a number of known K protein partners. In addition to recruiting PKCδ, this domain also mediates the binding of the transcriptional repressors Zik1 (2) and Eed (22), several tyrosine kinases (3), and the proto-oncoprotein Vav (6). It is likely that this domain recruits many other factors that are involved in signal transduction and gene expression. We designate this region as the KI domain, for K protein interactive domain.

Role of Ser302 on the in vitro Interaction of K Protein with PKCδ in the Presence or Absence of RNA—Ser302 is located within the highly interactive KI domain, in the middle of a short amino acid stretch that splits the two clusters of SH3-binding domains. The location of Ser302 suggests that this residue might play a role in the regulation of K protein interaction with PKCδ and/or other partners. To test this possibility, beads bearing either wild-type GST-K protein or GST-K protein with mutated Ser302, GST-K(S302A) or GST-K(S302E), were mixed with baculovirus expressed PKCδ and the binding was assessed as before by SDS-PAGE and Western blotting with an anti-PKCδ antibody. Results illustrated in Fig. 6A show that mutating Ser302 to either Ala or Glu did not alter the ability of K protein to recruit PKCδ. The in vitro interaction of K protein with many of its molecular partners can be regulated by cognate RNA, such as poly(C) RNA, or a cognate DNA, such as the αB motif (4, 21). Thus, we also tested whether mutation of Ser302 alters the affinity of K protein-PKδ complex when K protein is bound to poly(C). These results showed that poly(C) RNA abrogated the in vitro interaction of K protein with PKCδ independently of Ser302 and suggests that the ability of K protein to bind poly(C) is not altered by the mutation of Ser302.

Abrogation of the in vitro association between K protein and PKCδ by poly(C) is similar to the observations of other K protein partners that are recruited to K protein by the KI domain (21).

Although under present conditions mutation of Ser302 had no detectable effect on the in vitro interaction of K protein with PKCδ or on its ability to bind poly(C), this residue may play a role in the engagement of other K protein partners. For example, the protein Eed is recruited to K protein by the KI domain (22), an interaction that could be modulated by Ser302. 35S-Eed was mixed with GST-K, GST-K(S302A), or GST-K(S302E) beads that had been pre-equilibrated with or without RNA. The K protein-bound 35S-Eed was analyzed by SDS-PAGE and autoradiography. These results revealed that mutation of Ser302 to Ala diminished the ability of 35S-Eed to be recruited by K protein, and the Ser302 to Glu302 mutation had even more of a blocking effect on this association. These results suggest that the recruitment of some of the K protein partners may be regulated by phosphorylation of Ser302.

Tyrosine Phosphorylation of K Protein Modulates Its Interaction with PKCδ in Vitro and in Vivo—Phosphorylation of K protein by the tyrosine kinase Lck regulates K protein inter-

FIG. 5. Binding of PKCδ to K protein in vitro. 20 μl of glutathione beads, suspended in PKC binding buffer and bearing either full-length or one of the K protein mutants fused to GST (A) were mixed with 0.2 μl of baculovirus expressed PKCδ for 1 h at 4 °C. The binding reaction, the beads were washed twice with 1.0 ml of PKC binding buffer and once with washing buffer. Beads were boiled in SDS-loading buffer, the sample was divided into two equal aliquots, and the eluted proteins were separated by SDS-PAGE. One gel was stained with Coomassie (B), while proteins from the other gel were electrotransferred to Immobilon-P membrane. The blot was immunostained with polyclonal anti-PKCδ antibody (Santa Cruz Biotechnology, Santa Cruz, CA) (B, PKCδ). Molecular mass markers are shown in kDa.

FIG. 6. Analysis of the effects of Ser302 mutation on the binding of PKCδ (A) and Eed (B) in the presence or absence of RNA. 20 μl of glutathione beads bearing either wild-type full-length GST-K (GST-K) or GST-K with either Ser302 → Ala (GST-K(S302A)) or Ser302 → Glu (GST-K(S302E)) mutations were preincubated for 30 min (4 °C) in 100 μl of standard binding buffer containing either no RNA (lanes 1, 4, and 7) or 10 μg/ml of either poly(A) (lanes 2, 5, and 8) or poly(C) (lanes 3, 6, and 9). Beads were washed twice with 1.0 ml of washing buffer and once with 1.0 ml of PKC binding buffer. A, washed beads were resuspended in 100 μl of PKC binding buffer, and the bead suspension was mixed with 0.2 μl of baculovirus expressed PKCδ for 1 h (4 °C). Beads were washed twice with 1 ml of PKC binding buffer and once with 1 ml of washing buffer. Proteins were eluted by boiling in SDS-loading buffer. The eluted proteins were separated by SDS-PAGE in duplicate gels. One gel was electrottransferred onto Immobilon-P membrane and was blotted with a polyclonal anti-PKCδ antibody (PKCδ), whereas the other was stained with Coomassie (GST-K). 50% of PKCδ load was run in lane 10. B, washed beads, loaded with or without RNA, were resuspended in 100 μl of HKMT buffer. The bead suspension was mixed with 0.2 μl of baculovirus expressed PKCδ for 1 h (4 °C). Beads were washed twice with 1 ml of HKMT buffer and once with 1 ml of washing buffer. Proteins were eluted by boiling the beads in SDS-loading buffer, and the eluates were separated by SDS-PAGE. Gels were stained with Coomassie (GST-K, lower panel) and were autoradiographed (35S-Eed, upper panel). 50% of the amount of 35S-Eed used in pull-down assays was run in lane 10 (load).
FIG. 7. Tyrosine phosphorylation of K protein stimulates its association with PKCδ. A, 15 μl of glutathione beads bearing GST-K protein were incubated in 50 μl of Lck phosphorylation buffer for 2 h at 30 °C without (lane 1) or with (lane 2) 2 μl of recombinant baculovirus Lck. The phosphorylation reaction was terminated by washing the beads four times with 1 ml of HKMT buffer. 0.6 ml of HKMT buffer containing 6 μl of 35S-PKCδ synthesized in cell-free system was centrifuged at 12,000 × g for 5 min at 4 °C. 200 μl of the spun solution was added to each aliquot of the washed beads, and the bead suspension was mixed for 30 min at 4 °C. The beads were then washed four times with HKMT buffer, and the bound proteins were eluted by boiling in loading buffer. Eluted proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. After staining with amido black (lower insert), 35S-labeled proteins were visualized by autoradiography (middle insert). The membrane was immunostained (IS, upper insert) with monoclonal anti-phosphotyrosine antibody (PY99, 1:2000 dilution, Santa Cruz Biotechnology) and alkaline phosphatase-conjugated goat anti-mouse antibody (1:1000 dilution, Santa Cruz Biotechnology). B: Jurkat cells (400 × 106 at 1.0 × 106 cells/ml) were treated with 3.5 mM H2O2/0.1 mM Na3VO4. At the given time points, cells were lysed with IP buffer for 30 min on ice. Lysates were centrifuged for 30 min at 13,000 rpm at 4 °C. 100 μl of cell lysate was sonicated for 60 min (4 °C) with 3 μl of either preimmune (lanes 1–3) or immune (lanes 4–6) anti-K protein antibody 54 (aK) rabbit serum. After sonication, the samples were centrifuged for 5 min at 15,000 × g (4 °C), and the supernatants were added to 20 μl of protein A/G beads (Santa Cruz Biotechnology). The suspensions were mixed for 30 min (4 °C), then the beads were washed four times with 1 ml of IP buffer, and the proteins were eluted by boiling in 50 μl of loading buffer and resolved by SDS-PAGE. After electrotransfer to Immobilon-P membrane, immunostaining was done either with (lower insert) anti-K protein serum 54 (1:5000 dilution), alkaline phosphatase-conjugated anti-rabbit antibody (1:3000 dilution, Bio-Rad), or 5-bromo-4-chloro-indolyl-phosphatase/nitroblue tetrazolium phosphatase substrate (Kirkgaard & Perry Laboratories) or with (upper insert) anti-phosphotyrosine monoclonal (PY99) as in A (middle blot). C, 200 μl of lysates from given time points of H2O2/Na3VO4-treated Jurkat cells were sonicated for 2 h (4 °C) without (lane 1) or with (lane 2) 2 μl of recombinant baculovirus Lck. The gels also showed that tyrosine phosphorylation of K protein (lanes 1–3) greatly enhanced the phosphorylation of PKCδ (lanes 4–6) (Transduction Laboratories) or 50 μl of anti-Flag monoclonal antibody (aFlag, lanes 4–6) (Santa Cruz). After sonication, 20 μl of protein A/G beads were added to each sample. The suspensions were mixed for 2 h (4 °C), the beads were washed four times with 1 ml of IP buffer, and the proteins were eluted by boiling in loading buffer. Eluted proteins were resolved on SDS-PAGE, and after electrotransfer to Immobilon-P membrane, immunostaining of K protein was carried out with anti-K protein antibody 54 (aK) using the sandwich technique (upper insert). To assess the levels of PKCδ in the immunoprecipitates (middle blot), Immobilon-P membranes were immunostained with a monoclonal anti-PKCδ antibody (1:200 dilution) (Transduction Laboratories), alkaline phosphatase-conjugated goat anti-mouse antibody (1:1000 dilution) (Santa Cruz), and 5-bromo-4-chloro-indolyl-phosphatase/nitroblue tetrazolium. To assess tyrosine phosphorylation of PKCδ (lower insert) membranes were immunostained with anti-phosphotyrosine monoclonal antibody (1:200 dilution) (PY99) and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:2000 dilution) (Amersham Pharmacia Biotech) and developed with ECL (Amersham Pharmacia Biotech).

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action with a number of its molecular partners.2 We tested whether tyrosine phosphorylation also regulates the association of PKCδ with K protein. Glutathione beads bearing GST-K protein were incubated in Lck phosphorylation buffer with or without baculovirus Lck. After extensive washes, the beads were incubated with 35S-PKCδ synthesized in the cell-free system (Promega). The beads were washed again, and the bound proteins were eluted by boiling, separated by SDS-PAGE, and transferred to Immobilon-P membrane. Fig. 7A illustrates results from this experiment. Lck-mediated tyrosine phosphorylation of K protein (upper blot) greatly enhanced the in vitro binding of 35S-PKCδ to K protein (Fig. 7A, lanes 1 and 2, middle blot). The gels also showed that tyrosine phosphorylation of K protein shifted its electrophoretic mobility and the protein band became wider (Fig. 7A, compare lanes 1 and 2, bottom blot). The marked changes in the electrophoretic mobility indicate a significant change in K protein structure that coincides with strong recruitment of PKCδ to K protein. Not unexpectedly, the Lck-mediated phosphorylation of K protein has different effects on the interaction of K protein with different molecular partners. Although the K protein binding of PKCδ
Fig. 7A, as well as the binding of tyrosine kinases and Vav, is increased by tyrosine phosphorylation of K protein, the K protein binding of Zik-1 and Eed is blocked by tyrosine phosphorylation. Moreover, the K protein binding of elongation factor 1α and TATA-binding protein is not affected by K protein tyrosine phosphorylation.

The combination of H2O2/Na3VO4 stimulates tyrosine kinases in a myriad of cell types (38). To test the effects of tyrosine phosphorylation of K protein on in vivo binding of PKCδ, Jurkat cells were treated with H2O2/Na3VO4. At given time points shown in Fig. 7, cell lysates were prepared, and immunoprecipitations were carried out with either pre-immune or immune anti-K protein serum. After SDS-PAGE and electrotransfer, Immobilon-P membranes were immunostained with either anti-phosphotyrosine (Fig. 7B, lower blot) or anti-K protein (Fig. 7B, upper blot). Anti-phosphotyrosine immunostaining showed that treatment of Jurkat cells with the combination of these agents induced a transient increase in tyrosine phosphorylation of K protein; there was a low constitutive level of K protein tyrosine phosphorylation, an easily detectable increase after 15 min of treatment, and a decrease after 60 min (Fig. 7B, upper blot). As in vitro, the electrophoretic mobility of in vivo tyrosine phosphorylated K protein was slower (Fig. 7B, compare lanes 4 and 5, upper blot).

Next we tested whether PKCδ exists in a complex with K protein in vivo and, if so, whether this association is regulated by tyrosine phosphorylation. Immunoprecipitations were carried on cell lysates from Jurkat cells treated with H2O2/Na3VO4 using a monoclonal anti-PKCδ antibody. A monoclonal anti-Flag antibody was used as a control. Fig. 7C (upper blot) shows that K protein co-immunoprecipitated with PKCδ from cell lysates; a very low level of K protein co-immunoprecipitated with PKCδ from cell lysates of untreated cells (lanes 1); there was a large increase in co-immunoprecipitated K protein after 15 min of treatment (lane 2) and a significant decrease at 60 min (lane 3). These results suggest that in vivo there is a low constitutive level of PKCδ binding to K protein, an association that is greatly enhanced by treatment of cells with H2O2/Na3VO4. To assess the levels of immunoprecipitated PKCδ and to determine whether there is tyrosine phosphorylation of PKCδ, anti-PKCδ immunoprecipitates from Jurkat cell lysates were analyzed by SDS-PAGE and anti-PKCδ (Fig. 7C, middle blot) and anti-phosphotyrosine (Fig. 7C, bottom blot) immunostaining. These results showed that the amount of PKCδ immunoprecipitated from cell lysates progressively decreased after the treatment and that the electrophoretic mobility of PKCδ was slower (compare lanes 1–3). The amount and the electrophoretic mobility of the immunoprecipitated PKCδ reflect accurately the levels of PKCδ found in these cell lysates by Western blotting (data not shown). Although the decrease in PKCδ levels in cell lysates may reflect translocation of this enzyme to the particulate fraction and/or proteolytic cleavage, the slower electrophoretic mobility may result from tyrosine phosphorylation of PKCδ. Because the in vitro binding of PKCδ to K protein is increased by tyrosine phosphorylation of K protein (Fig. 7A) and because treatment of Jurkat cells with H2O2/Na3VO4 stimulates tyrosine phosphorylation of K protein (Fig. 7B), the enhanced association of PKCδ with K protein in vivo is likely the result, at least in part, of tyrosine phosphorylation of K protein. Although in vitro the increased binding of PKCδ to tyrosine phosphorylated K protein does not require tyrosine phosphorylation of PKCδ (Fig. 7A), treatment of cells with H2O2/Na3VO4 stimulates tyrosine phosphorylation of this enzyme (Fig. 7C, lower blot), a modification that may contribute to the enhanced PKCδ-K protein association. Regardless of the specific mechanisms responsible for the enhanced association, these results illustrate that the binding of PKCδ to K protein is regulated in response to changes in the extracellular environment.

**Poly(C) RNA Disrupts the Native K Protein-PKCδ Complex—**The above results demonstrate that the in vitro complex formation between recombinant K protein and PKCδ is blocked by poly(C) RNA (Fig. 6), which tenaciously binds K protein. Next we tested whether cognate RNA can disrupt the native PKCδ-K protein complex. Proteins from cytoplasmic extracts were precipitated with either pre-immune or immune anti-K protein serum and protein A/G beads. After a round of washing, proteins were eluted from the beads with either PKC buffer alone or PKC buffer containing either poly(A) or poly(C) RNA. Eluates were then analyzed by SDS-PAGE followed by Western blotting with a monoclonal anti-PKCδ antibody. Immunostaining revealed that PKCδ was eluted from beads bearing anti-K serum but not from the pre-immune beads and that the amount of eluted PKCδ was the highest with poly(C) RNA (Fig. 8). This finding suggests that in vivo the cognate nucleic acids have the potential to regulate the PKCδ-K protein association.

Along with the observation that tyrosine phosphorylation modulates the association between K protein and PKCδ (Fig. 7), the effect of poly(C) RNA on this interaction (Figs. 6 and 8) provides further evidence that the association between the two proteins is regulated.

**DISCUSSION**

K protein has previously been shown to be phosphorylated in vivo, both constitutively and inductively, by serine/threonine kinases (5, 23). Until now, casein kinase II was the only kinase known to phosphorylate K protein (24, 39). In the present study, we demonstrate that K protein forms a complex with PKCδ (Figs. 6–8) and can serve as its substrate (Figs. 1 and 2). K protein is not a specific PKCδ substrate because it can also be phosphorylated by PKCζ, β, and δ (Fig. 3). In addition to the conventional (α, β, and γ) and novel PKCs (δ, ε, η, and ι), K protein may also be a substrate for the atypical PKCs (ζ and λ). In that regard, it is notable that hnRNP A1 is a substrate for, and binds to, PKCζ (40).

We have identified Ser302 as a major site of phosphorylation by PKCζ (Fig. 2) and by PKC (α, β, and γ) (Fig. 3) in vitro. This site is also phosphorylated in vivo in response to treatment of cells with PMA (Fig. 4), suggesting that the in vivo phospho-
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Ser302 may regulate cross-talk among factors that are simultaneously engaged in K protein. For example, Src-family of tyrosine kinases are activated, phosphorylating sites in conjunction with Ser302 may play a role in modulating the binding of PKCδ to K protein. Moreover, phosphorylation of Ser302 may regulate cross-talk among factors that are simultaneously engaged by K protein. For example, Vav binds K protein and is tyrosine phosphorylated in response to cytokines (41). Phosphorylation of Vav may occur in the context of K protein simultaneously engaging Vav and a tyrosine kinase via the two clusters of SH3-binding domains (3). If so, it is conceivable that the juxtaposition of Vav and its tyrosine kinases may be altered by PKCδ-mediated phosphorylation of K protein Ser302. Such a model could be applied to many of the K protein molecular partners.

K protein binds PKCδ with high enough affinity (Figs. 1 and 6) to allow the two proteins to exist as detectable complexes in vivo (Figs. 7 and 8). What is the physiological relevance of the inducible nature of this association? First, the inducible binding is likely to ensure effective phosphorylation of K protein by PKCδ, especially in response to a changing extracellular environment. Second, the binding of PKCδ to K protein may link this enzyme to its targets or effectors that are concurrently present in the K protein microenvironment. Third, because K protein shuttles between the nucleus and cytoplasm (15), PKCδ could be co-transported to specific subcellular compartments by K protein. These three scenarios are not mutually exclusive, and there may be other physiological meanings of the PKCδ-K protein association. Whatever the physiological role(s) may be, the PKCδ-K protein binding could be modulated by changes in the extracellular environment that may include oxidative stress (Fig. 7, B and C) and acute phase reactions. The in vivo interaction between the two partners may be further regulated by cognate nucleic acids, such as specific RNA sequences. These findings, in conjunction with the previous observations that K protein phosphorylation is modulated by interleukin-1 (5), and the report that in intact organs K protein association with some of its molecular partners is modulated by an acute phase reaction (11), provide evidence that K protein function is regulated and responds to the needs of the cell in the face of a changing external environment.

Based on what is already known about K protein and its molecular partners, there are a number of specific scenarios that can be envisaged where PKCδ-K protein interaction may play a role in determining cellular response. For example, in response to cytokines, oxidative stress, or acute phase reaction, the Src family of tyrosine kinases are activated, phosphorylating and then binding to K protein. Tyrosine phosphorylation of K protein would then induce enhanced binding of PKCδ to K protein (Fig. 7). The simultaneous binding of a tyrosine kinase and PKCδ in the context of K protein would provide an opportunity for cross-talk between these enzymes. Tyrosine-phosphorylated and activated PKCδ could then dissociate from K protein and target other factors. Alternatively, K protein may provide a platform that facilitates the ability of PKCδ to target those substrates that are simultaneously recruited by K protein. In regard to these models, a number of PKCs, including PKCδ (Fig. 7C), are tyrosine phosphorylated, a modification that renders them phospholipid- and Ca²⁺-independent (42). Because the recruitment of PKCδ to K protein can be dramatically altered in vitro by cognate RNA (Figs. 6 and 8), the K protein-facilitated cross-talk between PKCδ and its targets and/or its effectors may be regulated by RNA in vivo. Moreover, K protein serving as a nucleic acid-interacting docking platform would facilitate molecular cross-talk at sites of active transcription, translation, and other processes involving RNA and DNA. The recruitment of PKCδ to K protein is likely to be just one example of a more general phenomenon involving the association of K protein with several members of the PKC family of enzymes. This is supported by the observations that PKCa, β, and γ phospholipase K protein (Fig. 3) and by a report that K protein binds PKCα in vitro (43). Moreover, because the yeast homologs of mammalian K protein and PKC have been shown to be functionally linked (44), the K protein-PKC interactions appear to be evolutionarily conserved in species as diverse as yeast and mammals.

PKCδ binds to a site within the highly interactive K protein region hereby designated as the KI domain (Fig. 5). Besides PKCδ, the KI domain binds the Src family of kinases (3), the proto-oncoprotein Vav (7), the transcriptional repressor Zik1 (21), the Polycomb group protein Eed (22), and likely many other factors. Within the KI domain these K protein partners may bind to the same or different sites. However, not all K protein partners bind to the KI domain. For example, TATA-binding protein binds K protein very strongly (8) through a region that is different from the KI domain. Other examples of KI domain-independent interaction includes the binding of RNA and DNA to the KH domains (19, 45). Moreover, although the transcriptional factor C/EBPβ binds to a domain contained in the N-terminal half of the molecule (11), an interleukin-1-responsive kinase binds to a domain in the vicinity of the C terminus (3).

What is the significance of the observation that many factors that interact with K protein are recruited by the KI domain? At least two models can be construed. The two dimerization domains may allow K protein to form higher order structures. If so, oligomerized K protein would contain a number of KI domains that could engage the same or different partners permitting uni- or multi-lateral cross-talk. It has been suggested that K protein may be involved in the transport of mRNA (15). Because K protein has the ability to shuttle between the nucleus and cytoplasm (15), it may not only serve to transport RNA, but it may also shuttle proteins. In that case, the KI domain may serve as a docking site for transport of these factors between the nuclear and cytoplasmic compartments. Whatever the role of the KI domain may be, the activity of this region toward some of the partners is regulated by growth factors, cytokines, and other extracellular stimuli that could exert their effect through the activation of PKCδ, Src-tyrosine kinases, and other enzymes that phosphorylate sites located within the KI domain.

In summary, we have shown that PKCδ binds and phosphorylates K protein. These observations broaden the range of K protein interactions. PKCδ targets Ser302, which is located in the middle of what appears to be a highly interactive KI domain. The ability of PKCδ to inducibly bind and phosphorylate K protein may serve not only to alter the activity of K protein itself, but K protein may also provide an avenue for PKCδ to engage in a cross-talk with other K protein molecular partners.

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in response to specific changes in the extracellular environment.

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Regulated Interaction of Protein Kinase Cδ with the Heterogeneous Nuclear Ribonucleoprotein K Protein

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